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Novel cell lines established from pediatric brain tumors

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Abstract

The paucity of cell culture models for childhood brain tumors prompted us to establish pediatric cell lines for use in biological experiments and preclinical developmental therapeutic studies. Three cell lines were established, CHLA-200 (GBM), CHLA-259 (anaplastic medulloblastoma) and CHLA-266 (atypical teratoid rhabdoid tumor, AT/RT). Consistent with an AT/RT origin, CHLA-266 lacked INI1 expression and had monosomy 22. All lines had unique DNA short tandem repeat “fingerprints” matching that of the patient’s tumor tissue and were adherent on tissue culture plastic, but differed in morphology and doubling times. CHLA-200 had a silent mutation in *TP53*. CHLA-259 and CHLA-266 had wild-type *TP53*. All three lines were relatively resistant to multiple drugs when compared to the DAOY medulloblastoma cell line, using the DIMSCAN fluorescence digital image microscopy cytotoxicity assay. RNA expression of MYC and MYCN were quantified using RT-PCR (Taqman). CHLA-200 expressed MYC, DAOY and CHLA-259 expressed MYCN, and CHLA-266 expressed both MYCN and MYC. CHLA-200 was only tumorigenic subcutaneously, but CHLA-259 and CHLA-266 were tumorigenic both subcutaneously and in brains of NOD/SCID mice. Immunohistochemistry of the xenografts revealed GFAP staining in CHLA-200 and PGP 9.5 staining in CHLA-259 and CHLA-266 tumors. As expected, INI1 expression was lacking in CHLA-266 (AT/RT). These three new cell lines will provide useful models for research of pediatric brain tumors.

Keywords

Pediatric anaplastic astrocytoma; AT/RT; Medulloblastoma; Cell lines; Multi-drug resistance; Xenograft

Introduction

Tumors of the central nervous system (CNS) comprise 22% of all malignancies occurring in children up to 14 years of age and 10% of tumors occurring among 15–19-year-olds, making them the most common solid tumor in children, and second in incidence only to leukemias [1]. With the improved treatment of leukemias, brain tumors are now the leading cause of death from childhood cancer. Among pediatric brain tumors, astrocytomas and medulloblastomas/embryonal tumors are the most common, accounting for 52 and 21%, respectively [2, 3]. Atypical teratoid/rhabdoid tumor (AT/RT), first described in 1996, is a highly malignant and increasingly-recognized CNS tumor that primarily occurs in very young children and typically has biallelic deletion and/or mutations in the *INI1/hSNF5* gene [4, 5].

Advances in neurosurgery, radiation therapy, and chemotherapy are responsible for a considerable improvement in long-term survival of children with brain tumors, but a significant proportion of patients continue to die of their disease [6]. One reason may be an intrinsic or acquired resistance to radiation and chemotherapy, i.e. that many brain tumors could intrinsically manifest a multidrug resistance (MDR) phenotype [7]. Tumor cell lines derived from CNS tumors are essential tools for studying the biology of the disease, for understanding mechanisms of resistance to therapy, and for carrying out preclinical therapeutic testing. However, to date only few continuous pediatric brain tumor cell lines have been established. Here we report the characterization of three new pediatric brain tumor lines: CHLA-200 (GBM), CHLA-259 (medulloblastoma) and CHLA-266 (atypical teratoid/rhabdoid tumor).

Materials and methods

Brain tumor tissue and cell line establishment

Between year 1996 and 2004, 161 clinical samples were received. Five cell lines were successfully established, and of them three grew well, and are described here. Tumors were obtained at surgery except one that was obtained at autopsy approximately 2 h after death (Table 1). Informed consent was obtained according to institutionally-approved protocols. Tissues were minced (cross scalpels) in a petri dish and cultured in 12.5 cm² filter-top cell culture flasks in Iscoves DMEM medium supplemented with 20% fetal bovine serum, 4 mM L-glutamine and 0.1% ITS stock solution (ITS = insulin, selenium and transferrin, Mediatech, VA). Gentamicin (50 µg/ml) was used for the initial few weeks of culture and then withdrawn to facilitate detection of mycoplasma. Cells were detached from the flask by using Puck's Saline with 1 mM EDTA and routinely cultured in a humidified incubator at 37°C in 5% carbon dioxide + 95% air. The three cell lines generated have been cryopreserved at low passages and propagated for at least 50 serial passages. Although the cell lines were established and grown routinely in serum containing medium as described above, the cell lines could also be grown as spheroids in serum-free Neurobasal®-A Medium + B-27 and N-2 supplements (Invitrogen, Carlsbad, CA), supplemented also with epidermal and fibroblast growth factors (10 ng/ml final concentration), (Promega, Madison, WI), and 2 mM L-glutamine (Mediatech, Inc, Manassas, VA).

Cell growth

Cells (10⁵ cells) were plated in triplicate in 25 cm² plastic tissue culture flasks. Cells were counted every 24 or 48 h after the initiation of cell culture. Cell population doubling time was determined using the following equation: doubling time (hours) = $0.693 (t - t_0) / \ln (N_t - N_0)$, where t_0 is the time when exponential growth began, t is time in hours, N_t is cell number at time t , and N_0 is cell number at t_0 .

Drugs and chemicals

Cisplatin (CDDP), fenretinide (4-HPR), melphalan (L-PAM), topotecan (TPT), and vincristine (VINC) were obtained from the Developmental Therapeutics Program, National Cancer Institute (NCI) (Bethesda, MD). 4-hydroperoxycyclophosphamide (4-HC) was kindly provided by Susan Ludeman at Duke University, North Carolina. Etoposide (ETOP) was obtained from Bristol-Myers Squibb Co. (Princeton, NJ). Fluorescein diacetate (FDA) was purchased from Eastman Kodak Company (Rochester, NY) and eosin Y from Sigma Chemical Co. (St. Louis, MO).

Genotyping of cell lines

We confirmed cell line identity by comparing each cell line to the original tumor tissue using short tandem repeat (STR) genotyping [8]. We used the AmpFISTR Identifier PCR Amplification kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommendations to amplify genomic DNA. The product was separated on a 3100-Avant Genetic Analyzer (ABI) and the results were analyzed using GeneMapper ID v. 3.2. software (ABI). Loci examined were 15 STR loci: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA, and the gender-specific locus amelogenin (<http://www.cogcell.org/clid.shtml>).

TP53 sequencing

Exons 5–10 of *TP53* in the three brain tumor cell lines were sequenced at University of Southern California Norris Comprehensive Cancer Center using the dye-based BigDye

V3.1TM method, where 3'-fluorescent-labeled dideoxynucleotides (dye terminators) are incorporated into DNA extension products (cycle sequencing) [9]. DNA sequencing was performed on ABI 3730 DNA Analyzer (Applied Biosystems, Forster City, CA). Primers were synthesized at Integrated DNA Technologies (Coralville, IA). For primer sequences see data archive at www.COGcell.org.

Western blot analysis

After incubation with or without L-PAM for 16 h, cells were detached, collected, lysed in RIPA lysis buffer (Upstate, Lake Placid, NY) containing 4 µl/ml aprotinin, 15 µl/ml PMSF and 40 µl Protease Inhibitor Cocktail (Sigma, St. Louis, MO), sonicated briefly, and centrifuged 10 min at 12,000×g. Cell lysates (20 µg/lane for p53 and MDM2 blots and 40 µg/lane for p21 blots) were resolved on a 4–20% continuous gradient Tris–glycine gel, transferred to nitrocellulose membrane (Whatman, Brentford, UK), and incubated with primary antibodies: Mouse-anti-human-p53 (BD Biosciences, CA), dilution 1:1,000; rabbit-anti-human-MDM2 (Santa Cruz, CA), dilution 1:500; goat-anti-human-p21 (Santa Cruz, CA) dilution 1:200 and HRP-labeled secondary antibodies (Santa Cruz, CA). Immunoblots were visualized by ECL chemiluminescence (Pierce Biotechnology, Rockford, IL). Densitometry analysis of western blots was performed using ImageJ version 1.63 (NIH, MD) and calculated using β-actin as a reference.

Cytotoxicity assay

Cytotoxicity was determined using the DIMSCAN assay system, which has a dynamic range of four logs of cell kill [10, 11]. The range of drug concentrations we used approximated clinically-achievable plasma levels, but may over-model the limited levels achievable in the CNS [9, 12, 13]. CHLA-200 and DAOY (fast growing) were plated at 3,000 cells/well into 96-well plates; CHLA-266 cells (intermediate) were plated at 6,000 cells/well, and CHLA-259 cells (slow growing) were plated 8,000 cells/well. All cell lines were seeded in 150 µl of complete medium per well. Cells were cultured in atmospheric oxygen (21% O₂) + 5% CO₂ and were allowed to attach 1 day before addition of cytotoxic drugs in complete medium in replicates of 12 wells/condition. To measure cytotoxicity, FDA and eosin-Y (the latter to quench background fluorescence) were added to the 96-well plate (final concentration, 10 µg/ml) and incubated for 30 min. Total fluorescence per well (after digital thresholding to further eliminate background fluorescence) was then measured using a DIMSCAN system and results were expressed as the survival fraction of treated cells compared with control cells. The concentration of drug that was cytotoxic and/or growth inhibitory for 90% of cells (IC₉₀) was calculated using the software “Calculusyn” (Biosoft, Cambridge, UK).

Quantitative real-time reverse transcriptase PCR

Primers and probes were designed using Primer Express (version 1.5, PE Biosystems) software. For primer sequences see data archive at www.COGcell.org. Total RNA was extracted from the cells using TRIzol (Invitrogen, Carlsbad, CA). All quantitative real time RT-PCR reactions were performed by Abi 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Forster City, CA). Every reaction included Master mix reagents (TaqMan one step RT-PCR Master Mix Reagents Kit, Applied Biosystems, Forster City, CA), 200 nM sense primer, 200 nM antisense primer, 100 nM TaqMan probe and 5 µl RNA sample (50 ng). TaqMan PCR data were analyzed using Sequence Detector (V1.7, Applied Biosystems, Forster City, CA). RNA isolated from a fibroblast cell line (CRL-2076, ATCC) and from the SMS-KCNr and CHLA-20 neuroblastoma cell lines were used as controls [9]. The mRNA level of each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Tumor implantation

Work was performed according to a protocol approved by the Children's Hospital Los Angeles Institutional Animal Care and Use Committee. Mice, 4–6-week-old female NOD/SCID (NOD/LtSz-scid/scid) and *nu/nu* were maintained under filter air barrier conditions and sterilized food and water. For intracranial injection, NOD/SCID mice were anesthetized and the head was fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) using ear bars [14, 15]. A small hole was made into the skull 2.0 mm lateral and 0.5 mm anterior to the bregma using a 27G needle. Tumor cells (2×10^5 cells) in 2 μ l IMDM without supplements were injected into the right caudate/putamen to a depth of 3.3 mm through a 30G needle at a rate of 0.1 μ l/min using a micro-injection system (Harvard Apparatus, MA). The needle was retained in place for additional 5 min and then was withdrawn slowly over 5 min. Mice were monitored daily for signs of distress and intracranial tumor size was followed by magnetic resonance imaging (MRI) at 1–3 week intervals, depending on the size of the tumor at prior MRI and the condition of the mice. Mice were sacrificed when the tumor reached a diameter of 4–5 mm or when the mice displayed signs of distress, whichever occurred earlier. For subcutaneous injection, 15×10^6 tumor cell and matrigel mix were injected s.c. into 4–6-week-old female athymic (*nu/nu*) mice between the shoulder blades.

Brain tumor imaging

MRI was performed using a 7 Tesla Bruker Pharmascan Instrument (Bruker, Ettlingen, Germany) using a 19 mm radiofrequency coil as previously described [16]. Fifteen to 20 min prior to scanning, mice were injected intraperitoneally with 100 μ l gadopentate dimeglumine (Magnevist, Bayer HealthCare Pharmaceuticals, Wayne, NJ) for tumor enhancement. Before scanning, mice were sedated with 5% isoflurane and held in an anesthetic plane with 1.5–2% for the entirety of the scans. Scans were performed using the following parameters: TR = 3076.5 ms, TE = 36.2 ms, number of excitations = 4, field of view = 2.6 cm, and final voxel sizes of $0.100 \times 0.100 \times 0.670$ mm slice thickness, with contiguous slices. Resultant images were visualized using the Bruker Pharmascan software and largest tumor axial dimensions were measured.

Histological and immunohistological examination

Mouse brains were removed under deep anesthesia following vascular perfusion with PBS followed by buffered 4% paraformaldehyde. Half of the brain was snap frozen in OCT and the other half fixed in 4% buffered paraformaldehyde for paraffin embedding. Sections (6 μ m) were cut and stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed using an automatic stainer BenchMark[®] (Ventana Medical Systems, AZ) according to manufacturer's instructions. The antibodies used were as follows: synaptophysin (DAKO, Glostrup, Denmark, 1:50), PGP 9.5 (Accurate Chemical, NY, 1:400), GFAP (Ventana Medical Systems, AZ, prediluted), vimentin (Ventana Medical Systems, AZ, prediluted), INI1 (BD Biosciences, CA, 1:200).

Statistical analysis

Each experiment was done using at least three replicates or performed three times unless indicated otherwise. In vitro cytotoxicity data were analyzed and graphed using the DIMSCAN Data Analyzer Program, and Sigmaplot 2000. For qRT-PCR gene expression data, the error bars represent SD and were calculated using Sigmaplot 2000. Results with a *P* value <0.05 were considered significant.

Results

Cell line morphology and growth characteristics

The paucity of cell culture models for childhood brain tumors prompted us to establish pediatric cell lines for use in biological and preclinical therapeutic studies. We established three pediatric brain cell lines summarized in Table 1. CHLA-200 was generated at autopsy from a recurrent glioma that at diagnosis was a frontoparietal anaplastic astrocytoma and at autopsy (2 years 2 months later) was gliomatosis cerebri consisting of GBM in the parietal lobe (where sample for the cell line was obtained), diffuse astrocytoma in the hemispheres, brainstem and cerebellum, and anaplastic ependymoma in the thalamus. CHLA-259 was an anaplastic medulloblastoma and CHLA-266 was an atypical teratoid rhabdoid tumor (AT/RT), both obtained from surgical specimens at diagnosis. Generated cell lines were examined by STR assay and matched their corresponding tumor specimens (Table 2). Figure 1 shows the morphology of the new cultured tumor cell lines. DAOY, a medulloblastoma cell line (ATCC) [17], was used for comparison. These new cell lines have been continuously cultured for more than 3 years and 50 passages and can thus be classified as permanent cell lines. In tissue culture all three lines were adherent. CHLA-200 (Fig. 1a) showed an elongated multipolar cell shape with long fibrillary processes. CHLA-259 and CHLA-266 (Fig. 1b, c) grew as a monolayer and were similar in morphology, with CHLA-259 morphology being less homogenous than CHLA-266. Doubling times of CHLA-200 (GBM), CHLA-259 (medulloblastoma), and CHLA-266 (AT/RT) were 54, 76, and 45 h, respectively (Fig. 1). As expected, in neuralbasal medium all three cell lines proliferated slower and grew as spheroids (data not shown).

Drug response profile

We next examined the cytotoxicity profiles of the new cell lines using several anti-neoplastic drugs that are used to treat pediatric brain tumors: CDDP, cyclophosphamide (in its active form 4-hydroperoxycyclophosphamide = 4-HC), ETOP, and VINC. For comparison we also tested TPT and L-PAM, that are often employed against neuroblastoma, a neural crest non-CNS tumor [9]. 4-HPR, an investigational synthetic retinoid, was also tested since it was reported to have preclinical activity in gliomas [18], medulloblastomas [19] as well as in rhabdoid tumor [20], a non-CNS tumor that is similar to AT/RT. Cytotoxicity assays were performed using a fluorescence microplate cytotoxicity digital image microscopy assay (DIMSCAN) [10]. CHLA-200 (GBM), which was obtained at autopsy from a heavily treated (VINC, carmustin, procarbazine, irradiation, and temozolomide), multiply-recurrent tumor was resistant to six of the seven tested drugs (Fig. 2; Table 3). CHLA-259, grown from an anaplastic medulloblastoma, which typically carries a relatively poor prognosis, was similar to DAOY in sensitivity to 4-HC, VINC and L-PAM, but more resistant to CDDP, ETOP, 4-HPR, and TPT (Fig. 2; Table 3). CHLA-266, that as an AT/RT is anticipated to be resistant to multiple cytotoxic agents, was indeed, resistant to six of the seven drugs we tested. Thus, this new panel of pediatric brain tumor cell lines may serve as useful models for studying these relatively drug-resistant childhood brain tumors.

p53 signaling

Since pathogenesis of many tumors is linked to mutations of *TP53*, we analyzed *TP53* exons 5–10 of the new cell lines for mutations using automated fluorescence dideoxynucleotide sequencing. CHLA-200 had a common polymorphism at codon 213, which does not affect coding of the p53 protein (CGA to CGG, both translate to arginine) [21]. CHLA-259 and CHLA-266 have wild-type *TP53*. Our laboratory previously studied p53 functionality in neuroblastoma cells using a luciferase reporter assay and also examination of expression of p53 targets by western blot following genotoxic stress and found that results were similar in the two approaches [9]. Therefore, here we assessed the functionality of the p53 pathway by

examination of changes in protein expression of p53 and its downstream targets p21 and MDM2 following 16 h exposure to the DNA damaging agent, L-PAM (10 μ g/ml). For comparison, we included in our analysis the *TP53*-mutated cell line, DAOY (single point mutation of *TP53* at base 725, resulting in substitution of phenylalanine for a cysteine at amino acid 242) [22]. As shown in Fig. 3, under baseline growth conditions CHLA-200, CHLA-259, and CHLA-266 expressed undetectable or low levels of p53, which increased 10-, 18-, and 5-fold, respectively, following exposure to L-PAM, suggesting functional signaling leading to p53. Following exposure to L-PAM, levels of p21, that normally increases in response to p53 increase and activation, did not increase in CHLA-200 (GBM), suggesting altered signaling downstream from p53 despite increased p53 protein expression. In CHLA-259 (medulloblastoma) and CHLA-266 (AT/RT) p21 did increase, suggesting functional p53-p21 axis. However, MDM2 expression (also a downstream target of p53) was not affected by L-PAM in any of the four cell lines tested, indicating an altered p53-MDM2 axis response. In the p53-mutated DAOY cells, levels of p53, p21, and MDM2 were unaffected by exposure to L-PAM, consistent with the report that DAOY cells lack functional p53. Thus, all three new cell lines had altered p53 signaling response despite CHLA-259 and CHLA-266 having wild-type *TP53* and CHLA-200 having a polymorphism that is thought to not affect the amino acid sequence of p53. The functional significance of these findings is not known at this time.

Oncogene mRNA expression

To characterize the three new cell lines we measured expression of *MYC* family genes by RT-PCR. Two well-characterized neuroblastoma cell lines, CHLA-20 and SMS-KCNR (both overexpress MYCN) were included for comparison, and fibroblasts were used as a reference against which expression was compared. *MYC* family genes *MYC* and *MYCN* have been widely studied due to their involvement in proliferation, differentiation, and potential role in tumorigenesis [23]. In neuroblastoma MYCN amplification is associated with poor prognosis [24]. In medulloblastoma MYCN and/or *MYC* can be amplified and highly expressed and are emerging as markers of poor prognosis [25–27]. Indeed, both medulloblastoma lines (CHLA-259 and DAOY) showed high MYCN but no increase in MYC expression. CHLA-266 (AT/RT) also had high expression of MYCN, but also had 3.6-fold $\pm$ 0.8 (SD; P = 0.0064) increase in MYC expression. CHLA-200 (GBM) on the other hand, had 3.6-fold $\pm$ 0.1 (SD; P < 0.0001) increase of MYC expression compared to fibroblasts, but no increase in MYCN (Fig. 4a).

Since many malignant cell lines express telomerase and maintain telomere length through an indefinite number of cell divisions [28] to avoid senescence, we also examined telomerase expression. The core telomerase enzyme consists of an RNA component (human telomerase RNA: hTR) and a catalytic protein component (human telomerase reverse transcriptase: hTERT) [29, 30]. As expected of continuous cell lines, the three new brain tumor cell lines and DAOY, as well as the two neuroblastoma cell lines, expressed high levels of both hTR and hTERT compared to the normal fibroblasts (Fig. 4b).

Intracranial growth

Last, the utility of tumor cell lines as pre-clinical models is greater if they can serve as in vivo tumor models in mice. To test this, we injected the three new cell lines both intracranially into the caudate/putamen nuclei of NOD/SCID mice, and subcutaneously in *nu/nu* mice [14, 15]. All three cell lines gave rise to subcutaneous tumors (data not shown). However, the time to detection of subcutaneous tumors of CHLA-200 was more than 4 months compared with CHLA-259 (43 days) and CHLA-266 (56 days). Importantly, CHLA-259 and CHLA-266, but not CHLA-200, also developed into tumors when injected intracranially, and were first detected by MRI on days 27 and 26, respectively (Fig. 5a).

Mean time to symptoms of mice with orthotopic tumors was 58 days $\pm$ 19 for CHLA-259 (n = 8) and 45 days $\pm$ 5 for CHLA-266 (n = 6).

Morphology of the mouse intracranial CHLA-259 and CHLA-266 tumors recapitulated the histology of the original patient tumor (Fig. 5b). For CHLA-259 (medulloblastoma), both the original tumor and mouse intracranial tumors were composed of densely packed primitive cells with round-to-oval hyperchromatic nuclei surrounded by scanty cytoplasm, as typically seen in medulloblastomas, and apoptosis was frequent (arrows). For CHLA-266 (AT/RT), both the original tumor and mouse intracranial tumors contained a population of pleiomorphic neoplastic cells. Typical rhabdoid cells (arrowheads) were seen which had round to lobulated eccentric nuclei, often with a prominent nucleolus, and cytoplasm that was eosinophilic or vacuolated. Cell borders were typically distinct and mitosis and apoptosis were present. Thus, in both tumors, the morphology of the mouse orthotopic tumors was similar to the morphology of the tumors resected from the patients.

Xenografts of the three new cell lines also represented the immunohistochemical characteristics of the original tumor lineage (Fig. 5c). The immunohistochemical stains of the intracranial (CHLA-259, CHLA-266) and subcutaneous (CHLA-200) tumors in the mice were as anticipated for these types of tumors: synaptophysin, an integral membrane glycoprotein in synaptic vesicles that is expressed in all neurons in the brain, was expressed diffusely in CHLA-259, but not in CHLA-266 and CHLA-200. In the normal brain synaptophysin only stained neuropil and neural outer membranes. PGP 9.5, that is expressed in neurons and in cells of the neuroendocrine system, was diffusely expressed in both embryonal tumors, CHLA-259 and CHLA-266, but not in CHLA-200. In the normal brain PGP 9.5 stained neuropil, nucleus, and the cytoplasm of normal neurons. GFAP (glial fibrillary acidic protein), that comprises glial filaments and is a glioma marker, was extensively expressed by CHLA-200 cells, but was only found in resident astrocytic filaments in the other tumors and the normal brain. Vimentin, an intermediary filament present in mesenchymal cells, endothelial cells and glia, was abundant in all three brain tumors. In normal brain vimentin was only expressed in the vascular wall structures. The tumor suppressor gene, *INI1*, a member of the chromatin remodeling complex SWI/SWF, is expressed in nuclei of all cells and is typically absent from rhabdoid and AT/RT tumors. Indeed, *INI1* was expressed all nuclei in the normal brain as well as in CHLA-259 and CHLA-200 tumors. However, CHLA-266 AT/RT showed no *INI1* staining in the tumor cell nuclei, with cells of a capillary structure in the tumor continuing to express it and serving as an internal control (Fig. 5). The karyotype of CHLA-266 demonstrated monosomy 22, suggesting that lack of *INI1* expression is due to loss of one allele of *INI1* and a mutation in the other allele (data not shown).

Discussion

Low passage primary cell cultures are frequently considered superior to continuous cell lines in brain tumor research. However, their availability, especially for cancers with low incidence, can be limiting to scientific progress. Moreover, when one desires permanent long term and stable manipulation of cells, this is hard to achieve with primary cultures, and primary cultures can be stressed from adaptation to the culture environment. Therefore, to allow timely research progress it is also necessary to utilize continuously growing cell lines. This is especially true for pediatric brain tumors, which are far less frequent than adult gliomas. Here we characterized three new continuous pediatric brain tumor cell lines that we now make available to the research community.

Although medulloblastoma is the most common malignant brain tumor of childhood, a relatively small number of human medulloblastoma cell lines have been generated thus far

[17, 31–34]. Existing pediatric GBM cell lines are even fewer [31, 35]. This is in marked contrast to other related primitive neuronal tumors such as neuroblastoma and Ewing's family tumors, where many cell lines and transplantable xenograft models have been successfully established. We have established three new cell lines from primary pediatric brain tumors: CHLA-200 (GBM, from gliomatosis cerebri), CHLA-259 (anaplastic medulloblastoma), and CHLA-266 (atypical teratoid/rhabdoid tumor, AT/RT), all of which grow continuously in cell culture and are tumorigenic in vivo (CHLA-259 and CHLA-266 were tumorigenic orthotopically in the brain and all three were tumorigenic subcutaneously). The establishment of CHLA-200 from a post-mortem sample suggests that such an approach can increase the number of post-chemotherapy brain tumor cell lines available for research, although these lines may behave differently compared to those obtained at diagnosis.

Successful therapy of malignant brain tumors requires multi-modality therapy. While single drugs are only transiently and partially effective, testing cell lines against the single agents can provide the initial information about their sensitivity to such drugs. Despite this, one needs to take into account that under tissue culture conditions the cells are out of their natural microenvironment and may thus show an altered response. The drugs we compared comprise three groups: (1) drugs used in established protocols against medulloblastomas and AT/RT (CDDP, cyclophosphamide in its activated form 4-HC, ETOP, and VINC) [12, 36–39], (2) drugs frequently used in our laboratory that are in protocols to treat neuroblastoma, a neural crest non-CNS tumor (topotecan, TPT and melphalan, L-PAM) [9], and (3) an investigational synthetic retinoic, 4-HPR, that has been reported to have preclinical activity in gliomas [18], medulloblastoma [19] and rhabdoid tumors [20], but had not been reported in AT/RT. DAOY medulloblastoma cells are frequently used as a model for testing drugs and we therefore used DAOY to compare responses of the new cell lines to the drugs.

CHLA-259 (anaplastic medulloblastoma) sensitivity to 4-HC and VINC was similar to DAOY, but CHLA-259 were less sensitive to CDDP and ETOP. CHLA-200 (GBM) and CHLA-266 (AT/RT) were all less sensitive to the four drugs (4-HC, CDDP, ETOP and VINC) compared to DAOY cells (Table 3). Both CHLA-266 (AT/RT) and CHLA-259 (anaplastic medulloblastoma) were generated from tumors at diagnosis that had not been exposed to chemotherapy before, indicating that they were inherently resistant to these drugs. CHLA-200 (GBM) that was isolated from a recurrent tumor that had previously been treated with VINC, not surprisingly showed marked resistance to VINC (Table 3). However, despite never being treated with cyclophosphamide, CDDP or ETOP, CHLA-200 was also resistant to them, consistent with GBMs being resistant to multiple chemotherapies. The last drug, an investigational synthetic retinoid, 4-HPR, was tested since it had been reported to have preclinical activity in gliomas [18], medulloblastoma [19] and rhabdoid tumors [20]. In rhabdoid tumors 4-HPR decreased cyclin D1, thus inhibiting rhabdoid tumor cell line proliferation in vitro [20]. Since AT/RT is similar to rhabdoid tumors in its molecular pathogenesis (lack of INI1) and high therapy resistance, this prompted us to investigate sensitivity of the AT/RT cell line, CHLA-266, to 4-HPR. In our experiments, 4-HPR had activity at clinically-relevant concentrations against CHLA-266 (AT/RT), CHLA-200 (GBM) and DAOY (*TP53*-mutated medulloblastoma), but not against CHLA-259 (anaplastic medulloblastoma). This is the first indication that AT/RT (CHLA-266) and DAOY cell lines are responsive to 4-HPR.

TP53 mutations are infrequent in pediatric CNS tumors (~10%) [40, 41]. *TP53* sequence was wildtype in CHLA-259 (anaplastic medulloblastoma) and CHLA-266 (AT/RT). CHLA-200 (GBM) only had a silent *TP53* polymorphism at exon 6 codon 213 (CGA to CGG) that is similar to a constitutional polymorphism reported in up to 10.8% of Italians [21] and is not thought to cause deficient p53 pathway signaling. Despite the normal amino acid sequence of p53 in the three lines, each of them showed at least some degree of

abnormal signaling. The expression of p53 in cancer cells with wt *TP53* is usually low and increases briefly upon exposure to DNA-damaging agents [42–44]. CHLA-259 and CHLA-266 were able to increase the level of p53 and its downstream target, p21 following L-PAM challenge, which suggests that signaling upstream of p53 and from p53 to p21 was functional. However, despite this increase in p53 in response to L-PAM, we did not observe concomitant changes in expression of MDM2, suggesting altered p53 response and possible defects in the downstream pathway. Interestingly, p53 pathway functionality in CHLA-200 cells was only manifest as increase in p53 expression in response to L-PAM, but failed to increase expression of p21 or MDM2, suggesting that p53 signaling was also defective in the CHLA-200 cell line. Interestingly, despite their lack of p53, DAOY cells were relatively more sensitive to most of the drugs we tested compared to the new cell lines.

In order to achieve immortality tumors need to maintain telomere length and telomere integrity [28]. In most cases, this is achieved through reactivation of a reverse transcriptase mechanism, by which telomerase adds TTAGGG units to telomeres. Cargioli et al. [45] have established permanent cell line models via transduction of hTERT and SV40 into early passages of temporary cell lines, which demonstrated the need for telomerase activity. Similar to our cell lines other glioma and medulloblastoma tumors express high level of hTERT and hTR [32, 46]. However, to date, this is the first report of hTERT expression in AT/RT tissues or cell lines.

Several reports have described MYC mRNA expression in medulloblastoma: 18 of 59 cases (31%) and 30 of 72 cases (42%) [47, 48]. Moreover, Stearns et al. [49] reported that MYC overexpression caused anaplasia in DAOY and UW228 medulloblastoma cell lines and tumors derived from them. Neither our medulloblastoma cell line, CHLA-259, nor DAOY expressed MYC. Hayashi et al. [50] described correlation between MYC expression and grade of astrocytoma. Accordingly, CHLA-200, originating from a high-grade glioma (GBM, WHO grade IV), did express MYC. Additionally, MYC expression was mildly increased in CHLA-266 (AT/RT), similar to another reported AT/RT cell line [51].

Genomic amplification and high expression of *MYCN* is often observed in high-risk poor-prognosis neuroblastoma [52–54]. Amplification and high expression of *MYCN* also occurs in CNS embryonal tumors (medulloblastoma, PNET, and AT/RT), and is associated with worse prognosis [25–27, 55]. CHLA-259, an anaplastic medulloblastoma, showed high MYCN expression, as did the AT/RT line, CHLA-266. We found no expression of MYCN in CHLA-200 (GBM), as expected.

In conclusion, we have established three continuous pediatric brain tumor cell lines, CHLA-200 (GBM), CHLA-259 (anaplastic medulloblastoma), and CHLA-266 (AT/RT). These cell lines showed multi-drug resistance and two of them were tumorigenic intracranially in immunocompromised mice. These new cell lines will provide useful models for studies of pediatric brain tumor biology and for preclinical testing of novel drugs for possible activity against multidrug resistant pediatric brain tumors. The three cell lines are available from the Children's Oncology Group Cell Line and Xenograft Repository (www.COGcell.org).

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Abbreviations

AT/RT	Atypical teratoid/rhabdoid tumor
VINC	Vincristine
4-HC	4-Hydroperoxycyclophosphamide
L-PAM	Melphalan
CDDP	Cisplatin
4-HPR	Fenretinide
TPT	Topotecan
ETOP	Etoposide
FBS	Fetal bovine serum
ITS	Insulin, selenium, and transferrin
GFAP	Glial fibrillary acidic protein
CNS	Central Nervous System

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Fig. 1. Morphology of pediatric brain tumor cell lines in log phase growth. Doubling time (DT) of each cell line is shown in the panel

Fig. 2. CHLA-200, CHLA-259 and CHLA-266 show higher resistance to most chemotherapy drugs tested as compared to DAOY cells. Cytotoxicity was analyzed after 4 days exposure using the DIM-SCAN assay to cisplatin (CDDP), cyclophosphamide (as 4-hydroperoxycyclophosphamide, 4-HC), etoposide (ETOP), melphalan (L-PAM), topotecan (TPT), vincristin (VINC), and ferentinide (4-HPR). For each drug concentration $n = 12$

Fig. 3. Melphalan (L-PAM) induced variable p53 expression and signaling in CHLA-200, CHLA-259, CHLA-266, and DAOY cell lines. Cells (1×10^6) plated in six well plates were incubated with L-PAM (6 $\mu\text{g/ml}$) or vehicle in 2 ml medium. After 16 h cells were collected, cell lysates (20 μg for p53 and MDM2 and 10 μg for p21) were resolved by SDS-PAGE and detected by western blotting. Fold increase in expression in presence of drug compared to control was calculated by densitometry using actin as loading control, and is denoted below the blots. This is a representative experiment of three with similar results

Fig. 4. The new pediatric brain tumor cell lines express telomerase and *MYC* genes. Quantitative RT-PCR of the three cell lines and DAOY: **a** mRNA expression of *MYC* and *MYCN* oncogenes, **b** mRNA of telomerase: hTR and hTERT. Bars represent means $\pm$ SD of triplicate samples in one representative experiment out of three experiments with similar results

Fig. 5. CHLA-259 and CHLA-266 form intracranial tumors in mice. **a** MRI images of tumors in mouse brains: *Left panel* CHLA-266, day 82, 2.1 × 2.0 mm tumor, *Right panel* CHLA-259, day 120, 1.9 × 2.4 mm tumor. **b** H&E stains of the original patient tumors (*top panels*) and the mouse brain tumors from orthotopic implantation of their respective cell lines (*bottom*). Original magnification is 4009. *Arrows* Apoptotic cells in CHLA-259, *Arrowheads* typical rhabdoid cells in CHLA-266. **c** Immunohistochemical staining for synaptophysin, PGP 9.5, GFAP, vimentin and INI1 of the mouse xenograft of CHLA-200 (subcutaneous), CHLA-266 and CHLA-259 (both intracranial) compared to normal brain

Table 1

Clinical features of the three malignant brain tumors used to establish the tumor cell lines

Cell line	Diagnosis	Age (years)	Phase of therapy	Location
CHLA-200	GBM	12	Autopsy (s/p multiple recurrences treated with chemotherapy and radiation)	Parietal lobe
CHLA-259	Anaplastic medulloblastoma	14	At diagnosis (before chemotherapy)	Posterior fossa (4th ventricle), no metastases
CHLA-266	Atypical teratoid/rhabdoid tumor	1.5	At diagnosis (before chemotherapy)	Posterior fossa with supratentorial metastases

Table 2

Short tandem repeats of the new cell lines show distinct genotypes

Locus	CHLA-200	CHLA-259	CHLA-266
D8S1179	10, 15	13, 14	10, 14
D21S11	29, 32.2	29, 30	29
D7S820	8, 12	10, 12	10, 12
CSF1PO	9, 11	10, 11	10, 11
D3S1358	14, 16	14, 15	15, 18
TH01	6	6, 8	6, 7
D13S317	11	9, 13	9, 10
D16S539	12	11	10, 12
D2S1338	18, 22	17, 18	20
D19S433	15	16	13, 14
vWA	17	17, 18	15, 17
TPOX	8	8, 10	8, 11
D18S51	15, 17	13	17, 19
AMEL	X, Y	X, Y	X
D5S818	9, 12	12	10, 12
FGA	21, 26	20, 26	23, 24

Table 3

CHLA-200, CHLA-259, and CHLA-266 show significant drug resistance compared to DAOY

IC ₉₀	4-HC (μg/ml)	CDDP (μg/ml)	ETOP (μg/ml)	VNC (ng/ml)	4-HPR (μM)	L-PAM (μg/ml)	TPT (nM)
CHLA-200	2.6 ± 2.0 *	4.6 ± 0.2 ***	2.1 ± 1.0 *	>50 ***	6.5 ± 0.2	12.2 ± 5.5 *	>1,000 ***
CHLA-259	1.7 ± 0.3	1.8 ± 0.8 *	0.6 ± 0.2 *	1.6 ± 1.4	>12 *	1.3 ± 1	72.2 ± 34.2 *
CHLA-266	6.9 ± 3.7 *	2.2 ± 0.5 **	>10 ***	>50 ***	3.5 ± 0.2	>20 **	>1,000 ***
DAOY	1.6 ± 0.3	0.3 ± 0.02	0.15 ± 0.03	2.2 ± 1.0	4.2 ± 1.2	1.7 ± 1.2	3.5 ± 2.0

Shown are IC₉₀ values calculated from experiments performed in Fig. 2. Experiments were performed at least three times and *P* values were assessed compared to DAOY

4-HC Activated cyclophosphamide, CPPD cisplatin, ETOP etoposide, VINC vincristine, 4-HPR fenretinide, L-PAM melphalan, TPT topotecan *P* values (compared to DAOY): NS: >0.05;

* *P* < 0.05,

** *P* < 0.01,

*** *P* < 0.001